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Description

The KRAS(G12D) Nucleotide Exchange Assay is a homogeneous assay designed for the screening and profiling of KRAS(G12D) antagonists/inhibitors by using BODIPY-GDP to monitor the GDP or GTP binding status. The KRAS(G12D) Nucleotide Exchange Assay Kit comes in a convenient 384-well format, with enough purified recombinant KRAS(G12D) labeled with BODIPY-GDP, GTP, assay buffer and additives for 400 enzyme reactions. The kit can be used with two different protocols for greater flexibility, either titrating the inhibitor at a fixed GTP concentration or titrating the GTP at a fixed inhibitor concentration.

BODIPY® FL GDP, is a mixed isomer in which the BODIPY® FL fluorophore has been attached to the 2' or 3' position of the ribose ring via a linker. BODIPY® FL dye is a green-fluorescent dye with similar excitation and emission to fluorescein or Alexa Fluor™ 488. It is characterized by a high extinction coefficient and high quantum yield and is relatively insensitive to pH changes. The dye has an excited-state lifetime of 5 nanoseconds or longer, ideal for fluorescence polarization–based assays.

Background

It is well established that RAS mutations are responsible more than 30% of human cancers. KRAS(G12D) is one of the KRAS mutations that is found frequently in pancreatic and colon cancers. Recent studies have led to the discovery of a small molecule called MRTX-1133 that locks KRAS conformation in the GDP-bound state, thereby blocking KRAS(G12D)-mediated signaling pathway. Compounds that affect the nucleotide exchange (GDP to GTP) reaction in KRAS could lead to a novel approach to the inhibition of tumor cell growth in KRAS(G12D)-driven tumors.

Mutations

G12D

Applications

Study enzyme kinetics and screen small molecular inhibitors for drug discovery and High Throughput (HTS) applications.

Supplied Materials

Catalog #	Name	Amount	Storage
100887	KRAS (G12D), Isoform A, His-Tag, BODIPY-GDP Loaded *	60 µg X 4	-80°C
79861-1	10 mM GTP	100 µl	-20°C
79862	2X KRAS buffer	5 ml	-20°C
	0.5 M DTT	200 µl	-20°C
	0.5 M EDTA	100 µl	Room Temp.
79961	Black, 384-well plate	1	Room Temp.
	Plate sealing film	1	

**The concentration of the protein is lot-specific and will be indicated on the tube.*

Materials Required but Not Supplied

Name	Catalog #
Fluorescent microplate reader capable of reading λex/em=470 nm/525 nm	-
Adjustable micropipettor and sterile tips	-

Stability

This assay kit will perform optimally for up to 6 months from date of receipt when the materials are stored as directed.

Safety

This product is for research purposes only and not for human or therapeutic use. This product should be considered hazardous and is harmful by inhalation, in contact with skin, eyes, clothing, and if swallowed. If contact occurs, wash thoroughly.

Assay Protocol

All samples and controls should be tested in duplicate.

A. GTP titration at a fixed inhibitor/antagonist concentration

1. Thaw 2x KRAS Nucleotide Exchange buffer, 0.5 M DTT, and thaw BODIPY-GDP loaded KRAS(G12D) on ice. Briefly spin the tube containing the protein to recover the full contents.

   Note: BODIPY-GDP loaded KRAS(G12D) is sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles. Do not freeze and re-use the diluted protein.

2. Prepare 1X KRAS buffer containing 1 mM DTT (e.g. for 4 ml 1X KRAS buffer, mix 2 ml of 2X KRAS buffer + 8 µl DTT (0.5 M) + 1,992 µl distilled water).

3. Prepare the Master Mix (20 µl): N wells X (5 µl of BODIPY-GDP loaded KRAS(G12D) + 2.5 µl of EDTA (25 mM) + 12.5 µl of 1X KRAS buffer containing DTT as prepared above). Add 20 µl of the Master Mix to all wells.

4. Prepare the Test Inhibitor (2.5 µl/well) at a concentration 10-fold higher than the desired final concentration. The final volume of the reaction is 25 µl.

   For example, MRTX-1133 was prepared at an intermediate concentration of 100 µM to obtain a final concentration of 10 µM.

   a) If the Test Inhibitor is water-soluble, prepare a 10-fold concentrated compound solution in 1x KRAS Nucleotide Exchange Buffer containing DTT. For the positive control, use 1x KRAS Nucleotide Exchange Buffer containing DTT (Diluent Solution).

   b) If the Test Inhibitor is soluble in DMSO, dissolve the Test Inhibitor at 100-fold the desired concentration in DMSO (*e.g.* if the testing concentration is 50 µM, prepare a 5 mM solution in 100% DMSO). Then dilute

the inhibitor 10-fold in 1x KRAS Nucleotide Exchange Buffer (containing DTT) to prepare the 10-fold concentrated intermediate solution (i.e. to test at a concentration of 50 µM, prepare a 500 µM intermediate solution by adding 5 µl of 5 mM inhibitor solution to 95 µl of KRAS buffer containing DTT). The concentration of DMSO in the intermediate solution is10%.

For the Positive control, prepare 10% DMSO in 1x KRAS Nucleotide Exchange Buffer containing DTT (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

Caution: Do not exceed 10% DMSO in the 10-fold intermediate solution.

5. Add 2.5 µl of Test Inhibitor to each well labeled Test Inhibitor. For control wells, add 2.5 µl of 1X KRAS buffer containing DTT in 10% DMSO if the inhibitor was dissolved in DMSO (Diluent solution without inhibitor). If the inhibitor is water soluble and was diluted in Assay buffer, add 2.5 µl of 1X KRAS buffer containing DTT to the control.

Component	**Positive Control**	**Test Inhibitor**
Master Mix	20 µl	20 µl
Test Inhibitor	-	2.5 µl
Diluent Solution (no inhibitor)	2.5 µl	-
GTP (0 mM – 1 mM)	2.5 µl	2.5 µl
Total	25 µl	25 µl

Note: The final concentration of DMSO in the assay should not exceed 1%.

Note: In the GTP titration experiment, a fixed concentration of the inhibitor is used. (e.g. for MRTX-1133, a fixed concentration of 10 µM final is recommended)

6. Centrifuge the plate to ensure all the components are mixed well and incubate the plate for 2 hours at room temperature.

7. Thaw 10 mM GTP and 0.5 M EDTA. Make 10-fold concentrated serial dilutions of GTP in distilled water from 0 mM to 1 mM. (These dilutions are 10-fold concentrations, so the final GTP concentrations in the reaction will be 0 to 100 µM). We recommend preparing 3-fold serial dilutions.

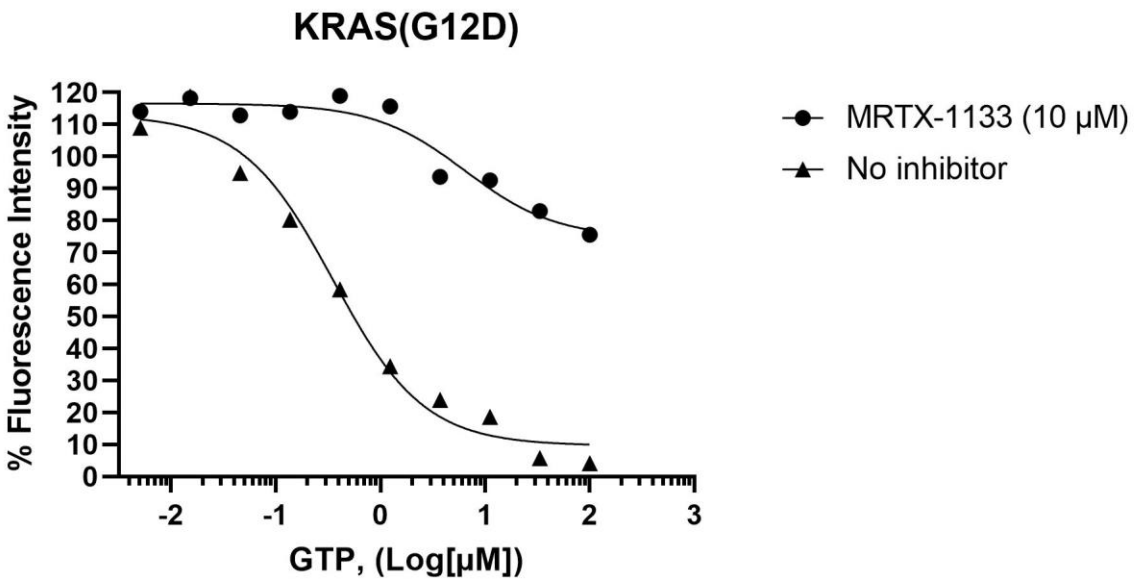
8. Dilute EDTA (0.5 M) in water to prepare 25 mM EDTA, e.g. to make 1 ml of 25 mM EDTA, mix 50 µl of EDTA (0.5M) with 950 µl of water.

9. After the 2-hour incubation, add 2.5 µl of the serially diluted GTP to the wells.

10. Initiate the reaction by adding 2.5 µl of 25 mM EDTA prepared above (final concentration of EDTA is 2.5 mM). Centrifuge the plate to ensure that all the components are mixed well and incubate the plate for 1 hour at room temperature.

11. After the 1-hour incubation, read the Fluorescence at λex470nm/em525nm.

Example of Assay Results:



F*igure 1: EDTA-mediated GDP-GTP exchange reaction of KRAS(G12D).* The assay was performed following the KRAS(G12D) Nucleotide Exchange Assay Kit protocol described above, in the presence or in the absence of 10 µM inhibitor MRTX-1133 and in the presence of increasing GTP concentrations

Results are representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

B. Inhibitor titration at a fixed GTP concentration

1. Thaw 2x KRAS buffer, 0.5 M DTT, and thaw BODIPY-GDP loaded KRAS(G12D) on ice. Briefly spin the tube containing the protein to recover the full contents.

   Note: BODIPY-GDP loaded KRAS(G12D) is sensitive to freeze/thaw cycles. Avoid multiple freeze/thaw cycles. Do not freeze and re-use the diluted protein.

2. Prepare 1X KRAS buffer containing 1 mM DTT (e.g. for 4 ml 1X KRAS buffer, mix 2 ml of 2X KRAS buffer + 8 µl of DTT (0.5 M) + 1,992 µl of distilled water)

3. Prepare the Master Mix (17.5 µl): N wells X (5 µl BODIPY-GDP loaded KRAS(G12D) + 12.5 µl 1X KRAS buffer prepared above).

4. Add 17.5 µl of the Master Mix to all wells.

5. Prepare the Test Inhibitor (2.5 µl/well). For the titration, prepare serial dilutions at concentrations 10-fold higher than the desired final concentrations. The final volume of the reaction is 25 µl.

6. a) If the Test Inhibitor is water-soluble, prepare serial dilutions in 1x KRAS Nucleotide Exchange Buffer (containing DTT), 10-fold more concentrated than the desired final concentrations. We recommend preparing 3-fold serial dilutions.

   For the positive control, use 1x KRAS Nucleotide Exchange Buffer containing DTT (Diluent Solution).

   b) If the Test Inhibitor is soluble in DMSO, prepare the Test Inhibitor at 100-fold the highest desired concentration in DMSO (*e.g*. if the highest testing concentration is 50 µM, prepare a 5 mM solution in 100% DMSO). Then dilute the inhibitor 10-fold in 1x KRAS Nucleotide Exchange Buffer (containing DTT) to prepare the highest concentration of the 10-fold concentrated intermediate solution (i.e. to test at maximum concentration of 50 µM, prepare a 500 µM intermediate solution by adding 5 µl of 5 mM inhibitor solution to 95 µl of KRAS buffer). The concentration of DMSO is now 10%.

7. Prepare serial dilutions of the Test Inhibitor at 10-fold the desired final concentrations using 10% DMSO prepared in 1x KRAS Nucleotide Exchange Buffer containing DTT (Diluent Solution) to keep the concentration of DMSO constant. We recommend preparing 3-fold serial dilutions.

   For the Positive control, prepare 10% DMSO in 1x KRAS Nucleotide Exchange Buffer containing DTT (vol/vol) so that all wells contain the same amount of DMSO (Diluent Solution).

   Caution: do not exceed 10% DMSO in the 10-fold intermediate solution.

8. Add 2.5 µl of the diluted inhibitor to the wells and centrifuge the plate to ensure all the components are mixed well. Add 2.5 µl of diluent solution without inhibitor in the control.

9. Incubate the plate for 2 hours at room temperature.

10. Thaw 10 mM GTP and 0.5 M EDTA. Prepare 10 µM GTP in distilled water [e.g. Dilute 10 mM GTP 10-fold in water (10 µl of GTP (10 mM) + 90 µl of water) to prepare 1 mM GTP, and dilute it 100 fold more to make a 10 µM solution (10 µl of GTP (1 mM) + 990 µl of water).

11. Dilute EDTA (0.5 M) in water to prepare 25 mM EDTA, e.g. to prepare 1 ml of 25 mM EDTA, mix 50 µl of EDTA (0.5M) with 950 µl of H2O.

12. Mix diluted GTP (10 µM) and EDTA (25 mM) at a 1:1 ratio.

13. After the 2-hour incubation, initiate the reaction by adding 5 µl of the GTP/EDTA solution prepared in step 10), and incubate the plate for 1-hour at room temperature.

14. After the 1-hour incubation, read the Fluorescence at λex470nm/λem525nm.

Component	**Positive Control**	**Test Inhibitor**
Master Mix	17.5 µl	17.5 µl
Test Inhibitor	-	2.5 µl
Diluent Solution (no inhibitor)	2.5 µl	-
GTP (0 mM – 1 mM)	5 µl	5 µl
Total	25 µl	25 µl

Example of Assay Results:

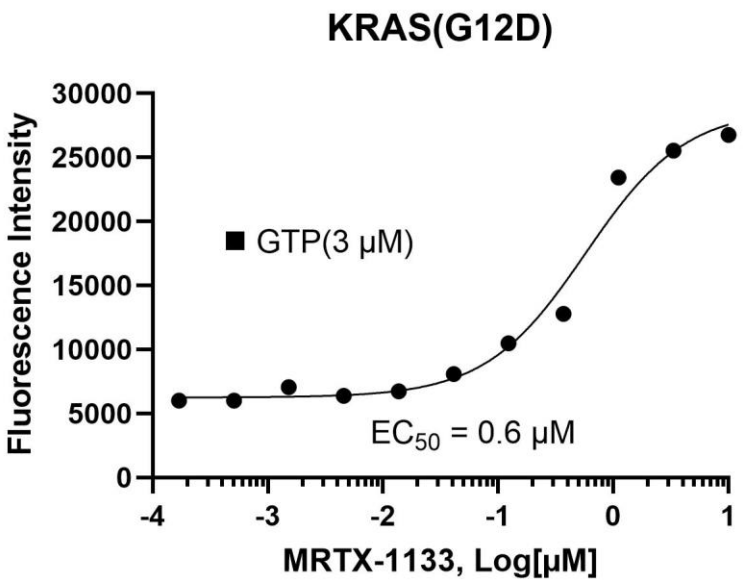


Figure 2: EDTA-mediated GDP-GTP exchange reaction of KRAS(G12D). The assay was performed following the KRAS(G12D) Nucleotide Exchange Assay Kit protocol described above, in the presence of a fixed GTP concentration and increasing concentrations of MRTX-1133.

Results are representative. For lot-specific information, please contact BPS Bioscience, Inc. at support@bpsbioscience.com

Troubleshooting Guide

Visit bpsbioscience.com/assay-kits-faq for detailed troubleshooting instructions. For all further questions, please email support@bpsbioscience.com.

References

1. Collins, Meredith A., *et al.* "Oncogenic Kras is required for both the initiation and maintenance of pancreatic cancer in mice." *The Journal of Clinical Investigation* (2012) 122.2: 639-653.
2. Engelman, Jeffrey A., *et al.* "Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and IK3CA H1047R murine lung cancers." *Nature Medicine* (2008) 14.12: 1351-1356.

Related Products

Products	*Catalog #*	*Size*
KRAS(G12C) Nucleotide Exchange Assay Kit	79859	384 rxns.
KRAS(G12C) Coupled Nucleotide Exchange Assay Kit (SOS1 mediated)	78004	384 rxns.
KRAS (G12D), Isoform A, His-Tag, BODIPY-GDP Loaded	100887	20 µg
KRAS (G12D), Isoform A, His-Tag, GDP-Loaded	101312	4 x 50 µg
KRAS (G12D), Isoform A, His-Tag	100623	100 µg
KRAS (G12C), Isoform A, BODIPY-GDP Loaded, His-Tag	100537	20 µg
KRAS (G13D), Isoform B, His-Tag	100479	100 µg
KRAS (G12R), Isoform B, His-Tag (Sf9-derived)	100825	100 µg
KRAS, Isoform B, His-Tag	11308	100 µg